

Weed seeds as nutritional resources for soil *Ascomycota* and characterization of specific associations between plant and fungal species

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Abstract Current interest in biological-based management of weed seed banks in agriculture furthers the need to understand how microorganisms affect seed fate in soil. Many annual weeds produce seeds in high abundance; their dispersal presenting ready opportunity for interactions with soil-borne microorganisms. In this study, we investigated seeds of four common broadleaf weeds, velvetleaf (*Abutilon theophrasti*), woolly cupgrass (*Eriochloa villosa*), Pennsylvania smartweed (*Polygonum pensylvanicum*), and giant ragweed (*Ambrosia trifida*), for potential as sources of carbon nutrition for soil fungi. Seeds, as the major source of carbon in an agar matrix, were exposed to microbial populations derived from four different soils for 2 months. Most seeds were heavily colonized, and the predominant 18S rRNA gene sequences cloned from these assemblages were primarily affiliated with *Ascomycota*. Further, certain fungi corresponded to weed species, regardless of soil population. Relatives of *Chaetomium globosum* (98–99% sequence identity) and *Cordyceps sinensis* (99%) were found to be associated with seeds of woolly cupgrass and Pennsylvania smartweed, respectively. More diverse fungi were associated with velvetleaf seeds, which were highly susceptible to decay. The velvetleaf seed associations were dominated by relatives of *Cephalophora tropica* (98–99%). In contrast to the other species, only few giant ragweed seeds were heavily colonized, but those that were colonized resulted in seed decay. The results showed that seeds could provide significant nutritional resources for

saprophytic microbes, given the extant populations can overcome intrinsic seed defenses against microbial antagonism. Further, weed species-specific associations may occur with certain fungi, with nutritional benefits conferred to microorganisms that may not always result in seed biodeterioration.

Keywords Weed seed bank · Seeds · Fungi · Seed decay · Seed-microbe association

Introduction

Weed infestations are ranked as the greatest problem in agricultural systems (Aref and Pike 1998), causing crop yield losses (estimated at 10% in the USA) that impact grower profitability. Attention towards reducing the dependency on herbicides has heightened interest in weed management strategies that combine more efficient use of herbicides with increased use of biologically based weed management methods (Kremer 1993; Buhler et al. 1998; Lutman et al. 2002; Forcella 2003; Baskin and Baskin 2006; Davis 2006; Gallandt 2006).

Buried seed reserves in the soil, usually referred to as the soil seed bank, play a key role in the population dynamics of individual plant species and are the major sources of future plant emergence (Fenner 1995). Among many annual weeds, seed production can range from thousands up to a million seeds per plant (Booth et al. 2003), with the resulting seed bank replenishment occurring each year. Upon entry into the soil environment, seeds may undergo several fates, including successful germination, entry into dormancy, predation, decay, and other mechanisms that result in loss of viability. The characteristics of natural seed bank dynamics have generally not included detailed studies

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of soil microorganisms and their role in mediating the outcome of seed fate, antagonistic or otherwise.

The susceptibility to decay of a variety of weeds can differ widely (Chee-Sanford et al. 2006; Davis et al. 2006; Davis 2007), with seed composition, structure, viability status, along with interactions with soil microbiota and the surrounding soil environment influencing a plant species' intrinsic mechanism of seed defense. For example, in addition to the seed coat of velvetleaf (*Abutilon theophrasti*) providing a physical barrier for protection of the endosperm, seeds produce a leachate of aqueous-soluble phenolic compounds that exhibit phytopathic, anti-fungal, and anti-bacterial activities (Kremer 1986; Paszkowski and Kremer 1988). Interestingly, while velvetleaf are known to produce long-lived seeds and persistent seed banks (Buhler and Hartzler 2001; Davis et al. 2005), seeds of this species are also observed to be highly susceptible to microbial-mediated decay. This abundance of reserved seed and the potential for microbial-mediated seed decay activities occurring in natural soil environments suggest that seeds may comprise a significant nutritional reservoir for soil microbial populations, dependent upon the conditions or activities that can overcome seed defense mechanisms to allow access to seed components.

The nutritional benefits associated with weed seed consumption in herbivory has been demonstrated (Dugan and Gumbmann 1990; Harrison et al. 2003, Lundgren et al. 2007), but the nutritional significance of annual seed deposition to natural soil microbial communities is yet unknown. Much of the focus on seed-microbe associations in the past has been directed in some fashion toward microorganisms and their role as pathogens, particularly with fungi (McKellar and Nelson 2003; Dent et al. 2004; Schafer and Kotanen 2004).

The ecological importance of fungi and their ubiquitous presence in soil environments is well recognized, with many species known for their role in nutrient cycling, plant productivity and health, and novel metabolic activity, along with numerous other ecological functions (Christensen 1989; Thorn 1997; Schulz et al. 2002; Vicente et al. 2003; Klein and Paschke 2004). Many well-known species of fungi have evolved interactions with plants, varying in outcomes that may be host-beneficial (e.g., mycorrhizae), host -antagonistic (e.g., pathogens), or neutral. Such relationships can also have profound effects on the associated microbe, influencing its survival and reproduction. To a lesser-known extent, fungal associations with plant diaspores are thought to play important roles in seed protection or germination (Klein and Paschke 2004).

The objective of this study was to determine if seeds of four broadleaf weed species problematic in corn and soybean cropping systems of the Midwest, velvetleaf (*A. theophrasti* M.), woolly cupgrass [*Eriochloa villosa*

(Thunb.) Kunth], Pennsylvania smartweed (*Polygonum pennsylvanicum* L.), and giant ragweed (*Ambrosia trifida* L.), could be utilized as primary sources of carbon for soil microbial populations. Based on preliminary observations that specific fungi might be associated with seeds, we focused on characterizing the assemblages of fungal growth after incubation on agar-containing mineral medium, where seeds presented the primary carbon source.

Materials and methods

Description of seeds and soils

Seeds of the broadleaf weed species velvetleaf, woolly cupgrass, Pennsylvania smartweed, and giant ragweed were harvested from plants, rinsed three times in sterile water, air-dried, and stored at 4°C in the dark before use. To ascertain the viability of seeds on average in the seed lots used, 100 seeds of each species were placed on 1.5% water agar medium, and germination (radicle emergence) was used as an indicator of seed viability. Soil from four different field Illinois locations in Champaign and Urbana were used as the sources of microbial inocula. All four soils were of silty clay loam texture (comprised of approximately 20% sand, 50% silt, 30% clay, organic matter content 4–7%, pH 6.1–6.2) typical of the region. Three of the soils (ASLOC, ASSWC, and ASSET) were from agricultural fields with a history of corn and soybean rotation, and one soil (USSEU) was from an undisturbed site with no known history of agronomic use.

Seed inoculation and examination of growth on seed surfaces

For each soil type, 1 g of soil was resuspended in 1× PBS (pH 8.0) and vortexed for 5 min at maximum speed, and then serially diluted 10,000-fold. An aliquot of 0.1 ml of the final diluted soil suspension was spread onto the surface of a phosphate-buffered (pH 7.0) agar medium consisting of ($\text{g}\cdot\text{l}^{-1}$): KH_2PO_4 , K_2HPO_4 , $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, NaSO_4 , NH_4Cl , trace metals, yeast extract (0.01%), and agar (1.5%) on standard Petri plates. Trace metals solution consisted of ($\text{mg}\cdot\text{l}^{-1}$) $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, H_3BO_3 , ZnCl_2 , $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, $\text{NiSO}_4\cdot 6\text{H}_2\text{O}$, $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, and $\text{NaMoO}_2\cdot 2\text{H}_2\text{O}$. Seeds of each individual weed species were embedded 0.5–1 cm apart into the inoculated agar surface. Because chemical or heat sterilization techniques described in the literature (e.g., bleach, autoclave, and irradiation) are not reliably effective at maintaining seed integrity and viability, background controls consisted of uninoculated agar medium containing seeds that had been rinsed thoroughly, as described above. Plates were sealed

with parafilm and incubated in the dark at 25°C for 3 months. Representative seeds were removed for microbial analysis and examined for obvious visible growth and with high magnification using an environmental scanning electron microscope (ESEM; Beckman Institute for Advance Science and Technology, Imaging Technology Group). Three representative seeds from each plate were tested for viability using the tetrazolium test (International Seed Testing Association 1985).

DNA extraction and PCR amplification

Seeds were individually placed into a sterile 2-ml tube containing 1 ml 1× PBS (pH 8.0). The seeds were vortexed for 15 min at maximum speed. Seeds were removed, and the cell suspension was centrifuged at 6,800×g for 15 min. Genomic DNA was extracted from the cell pellet using a phenol/chloroform extraction method (Tsai and Olson 1992) modified with an additional step of bead beating with 0.1- and 0.5-mm zirconium beads in 1 ml TE (pH 8.0) at maximum speed on a vortexer, followed by physical grinding of the mixture for 1 min using a stainless steel rod before chemical and enzymatic cell lysis. Purified DNA was resuspended in 5–10 µl TE (pH 8.0) buffer and stored at –20°C. DNA was quantified using band densitometry and compared to a known DNA standard after gel electrophoresis. DNA extraction was also performed on pooled batches of ten seeds from like species before microbial inoculum exposure using methods described above. Similarly, DNA extractions were also performed on pooled control (uninoculated) seeds incubated under the same conditions as the treated seeds.

The polymerase chain reaction (PCR) amplification of fungal 18S rRNA genes from genomic DNA extracted from each seed-associated microbial assemblage was carried out using primer set EF4/EF3 (Smit et al. 1999). PCR amplification was performed in a 20 µl reaction using the ExTaq PCR kit (Takara) with DNA template amounts ranging from ~10–20 ng. Amplification took place in a PTC-200 DNA Engine (MJ Research), and the program consisted of: 94°C for 10 min (1 cycle) followed by 94°C for 90 s, 50°C for 90 s, 72°C for 90 s (30 cycles), and 72°C for 10 min (1 cycle). PCR amplicons (~1.4 kb) were visualized and quantified by gel electrophoresis.

Restriction-enzyme digest analysis and cloning for sequencing of 18S rRNA genes

Clone libraries of amplified 18S rRNA gene products of each seed-associated fungal community were generated using the TOPO-TA cloning kit (Invitrogen). All white colonies were selected and screened directly for inserts by performing colony PCR with M13F/M13R primer set.

Further screening of clones was done by a nested PCR reaction with the EF4/EF3 primer set to confirm the presence of the correct fungal DNA insert.

The amplified PCR product was purified using the Qiagen PCR purification kit according to manufacturer's instruction. After confirmation of the cloned insert by amplification as described above, the PCR product (650–700 ng) was digested overnight with separate enzymes *RsaI*, *MspI*, *HaeII*, or *HhaI* (1 U enzyme per reaction) for amplified ribosomal DNA restriction analysis (ARDRA; Wu et al. 2006) to differentiate unique clones. ARDRA patterns for each cloned insert were grouped according to unique profiles. Based on ARDRA patterns, unique clones were selected for sequencing of the 18S rDNA gene. Plasmids containing the inserts were purified using the QIAprep spin miniprep kit (Qiagen). Twenty-nine 18S rDNA clones derived from seed surfaces were sequenced by the University of Illinois Keck Center for Biotechnology. Sequences were analyzed on-line using the Basic Local Alignment Search Tool (BLAST) family of programs of GenBank (Madden et al. 1996).

Phylogenetic analysis

Sequences were checked for chimeric artifacts using the CHIMERA-CHECK program of the Ribosomal Database Project (Maidak et al. 2001). Sequences were aligned with representative fungal sequences from GenBank using MacVector v. 7.2 software (Accelrys). A phylogenetic tree was constructed using the neighbor-joining method and the Jukes–Cantor algorithm for calculating the distance matrix.

Results

Microbial growth and characterization of cloned fungal 18S rRNA genes

Quantifiable amounts of DNA were isolated from all seeds; however, fungal 18S rRNA genes were not amplifiable in all samples. DNA yields were typically low, ranging in concentrations from 5–25 ng µl⁻¹. Bacterial 16S rRNA genes were amplified in all samples, indicating their presence in the microbial assemblages (data not shown). DNA recovered from individual seeds of velvetleaf ($n=16$), woolly cupgrass ($n=18$), and Pennsylvania smartweed ($n=16$) yielded amplified fungal 18S rRNA genes and were all used in subsequent cloning reactions. Only two seeds of giant ragweed yielded quantities of DNA suitable for subsequent analysis; however, amplification of fungal 18S rRNA genes did not yield any quantifiable products. Uninoculated seed controls and seeds analyzed before soil inoculum exposure did not yield amplified ribosomal gene

products or show visible growth on seed surfaces. In contrast, seeds showed obvious visible microbial growth after incubation with soil microbial populations (Fig. 1a–d). Seeds examined by ESEM further demonstrated the presence of dense and close cell associations (Fig. 1e–h). Even with no major exogenous carbon source (other than seed) added to the medium, some microbial growth was observed on the agar surface apart from direct contact with the seeds; however, it was clear that a high density of microbial biomass was associated directly with individual seeds.

A total of 105 clones were obtained, and digestion of the cloned ribosomal gene inserts with *RsaI* and *MspI* resulted in ARDRA patterns that effectively resolved at least 11 unique clones in total from seeds of both Pennsylvania smartweed and woolly cupgrass, and at least 10 unique

clones from seeds of velvetleaf. Further digestion with *HhaI* or *HaeIII* did not further distinguish the clones. Sequences of cloned 18S rRNA genes representing the unique ARDRA groups were selected for further sequencing and phylogenetic analysis.

Fungal 18S rRNA gene sequence analysis

Among the 29 clones selected for sequencing, all but 1 of the sequences matched closely to species that are members of the fungal phylum *Ascomycota* in the GenBank database (Table 1), with homologies ranging between 97 and 100% identity to known sequences in the NCBI database. Only one clone obtained from a velvetleaf seed matched most closely to *Actinomucor elegans* (99%; GenBank accession AF157119), belonging to the *Zygomycota* division. No sequence matches to species belonging to two other major fungal phyla *Basidiomycota* or *Chytridiomycota* were recovered. Non-fungal sequences were not obtained among the clones analyzed. A phylogenetic tree shows the distribution and relationships of the cloned fungal 18S rRNA genes obtained from the seed associations (Fig. 2).

There was a predominance of the ascomycete *Cordyceps sinensis* (99% identity to GenBank accession AB067700) among the eight clones obtained from Pennsylvania smartweed seeds after exposures to three of the four soil inocula. One clone from a Pennsylvania smartweed seed exposed to the soil inoculum derived from ASLOC matched closely to *Capronia pilosella* (98% identity to GenBank accession U42473). ARDRA analysis indicated at least five distinct subgroups of *Cordyceps sinensis* distributed among the similar clones.

Eight of the nine clones obtained from woolly cupgrass seeds exposed to inocula derived from USSEU, ASSET, and ASSWC were primarily matched to the ascomycete *Chaetomium globosum* (98–99% identity to GenBank accession AB048285; Table 1). One cloned fungal sequence from a seed exposed to ASLOC inoculum had the highest homology (99% identity) to *Cordyceps sinensis*. At least six distinct subgroups of *Chaetomium globosum* were in association with seeds based on ARDRA profiles.

Clones obtained from velvetleaf seeds had the highest sequence homologies to several genera of the *Ascomycota*, with more than half of the clones matching closely to *Cephalophora tropica* (98–99% identity to GenBank accession AB001111), with five of these clones representing distinct ARDRA subgroups (Table 1). *Cordyceps sinensis* (99% identity to GenBank accession AB067700) was present, represented by two distinct clones, and one clone matched closest to *Madurella mycetomatis* (99% identity to GenBank accession AF527811). The zygomycete *Aspergillus flavus* was the closest match (99% identity) to one unique clone. Visual examination of the velvetleaf

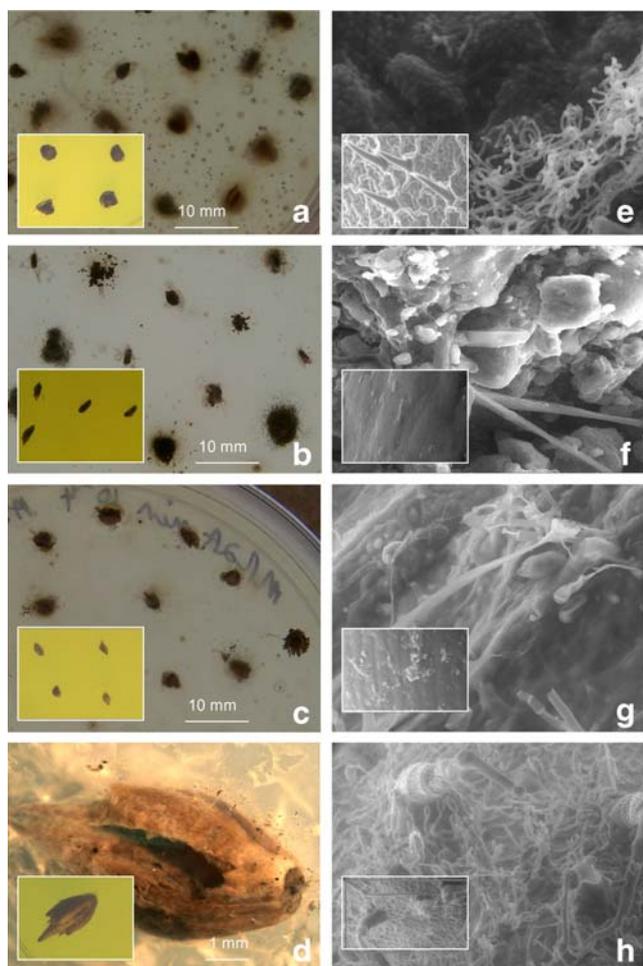


Fig. 1 Representative images of seeds after 2 months incubation after inundative exposure to soil microbial populations in a mineral agar matrix where seeds provided the sole carbon nutrition. Seeds of **a** velvetleaf, **b** Pennsylvania smartweed, **c** woolly cupgrass, and **d** giant ragweed, and sample views of corresponding microbial biomass on seeds viewed under high resolution ESEM of **e** velvetleaf, **f** Pennsylvania smartweed, **g** woolly cupgrass, and **h** giant ragweed. Inset micrographs show corresponding uninoculated seeds

Table 1 Clones from seed surfaces showing the nearest taxonomic identities from GenBank and the ARDRA group of each clone

Weed species	Soil inoculum	Clone ID	Closest fungal taxon ^a	Division	% Identity ^b	ARDRA group ^c		
Velvetleaf	USSEU	vv1-8B-2	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	B1		
		vv1-8B-3	<i>Cephalophora tropica</i> AB001111	Ascomycota	98	Hb		
		vv1-8B-9	<i>Cephalophora tropica</i> AB001111	Ascomycota	99	Kf		
		vv1-8B-12	<i>Cephalophora tropica</i> AB001111	Ascomycota	99	Jf		
		vv1-9B-1	<i>Madurella mycetomatis</i> AF527811	Ascomycota	99	Lx		
		vv1-9B-8	<i>Cephalophora tropica</i> AB001111	Ascomycota	99	Js		
		vv1-11B-1	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Bv		
	ASSET	vv1-11B-6	<i>Actinomucor elegans</i> AF157119	Zygomycota	99	Nw		
		vv1-11B-8	<i>Aspergillus flavus</i> AF548060	Ascomycota	99	Bx		
		vv1-11B-9	<i>Cephalophora tropica</i> AB001111	Ascomycota	99	Mv		
		vv1-11B-14	<i>Cephalophora tropica</i> AB001111	Ascomycota	99	nd		
		Woolly cupgrass	USSEU	wc-10B-4	<i>Chaetomium globosum</i> AY545725	Ascomycota	99	Ht
				wc-10B-5	<i>Chaetomium globosum</i> AY545725	Ascomycota	98	Iu
			ASSET	wc-10B-8	<i>Chaetomium globosum</i> AY545725	Ascomycota	99	Fb
wc-10B-9	<i>Chaetomium globosum</i> AY545725			Ascomycota	99	Hq		
wc-10B-11	<i>Chaetomium globosum</i> AY545725			Ascomycota	99	Fs		
ASLOC	wc-5B-5		<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Aj		
ASSWC	wc-7B-1		<i>Chaetomium globosum</i> AY545725	Ascomycota	99	nd		
	wc-7B-3	<i>Chaetomium globosum</i> AY545725	Ascomycota	99	nd			
	wc-7B-8	<i>Chaetomium globosum</i> AY545725	Ascomycota	99	nd			
Pennsylvania smartweed	USSEU	ps-2B-1	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Aa		
		ps-2B-2	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Bb		
	ASSET	ps-6B-2	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Aa		
		ps-6B-3	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Bb		
		ps-6B-4	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Bq		
		ps-7B-2	<i>Capronia pilosella</i> U42473	Ascomycota	98	Hr		
	ASSWC	ps-3B-6	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	Af		
		ps-3B-8	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	nd		
		ps-3B-10	<i>Cordyceps sinensis</i> AB067700	Ascomycota	99	nd		

nd Not determined

^a Shown with GenBank accession number

^b Cloned sequences used from this study for alignments and BLAST searches were 542–725 bases

^c ARDRA grouping is indicated by two-letter designations describing restriction enzyme digest patterns using *RsaI* (upper case) and *MspI* (lower case)

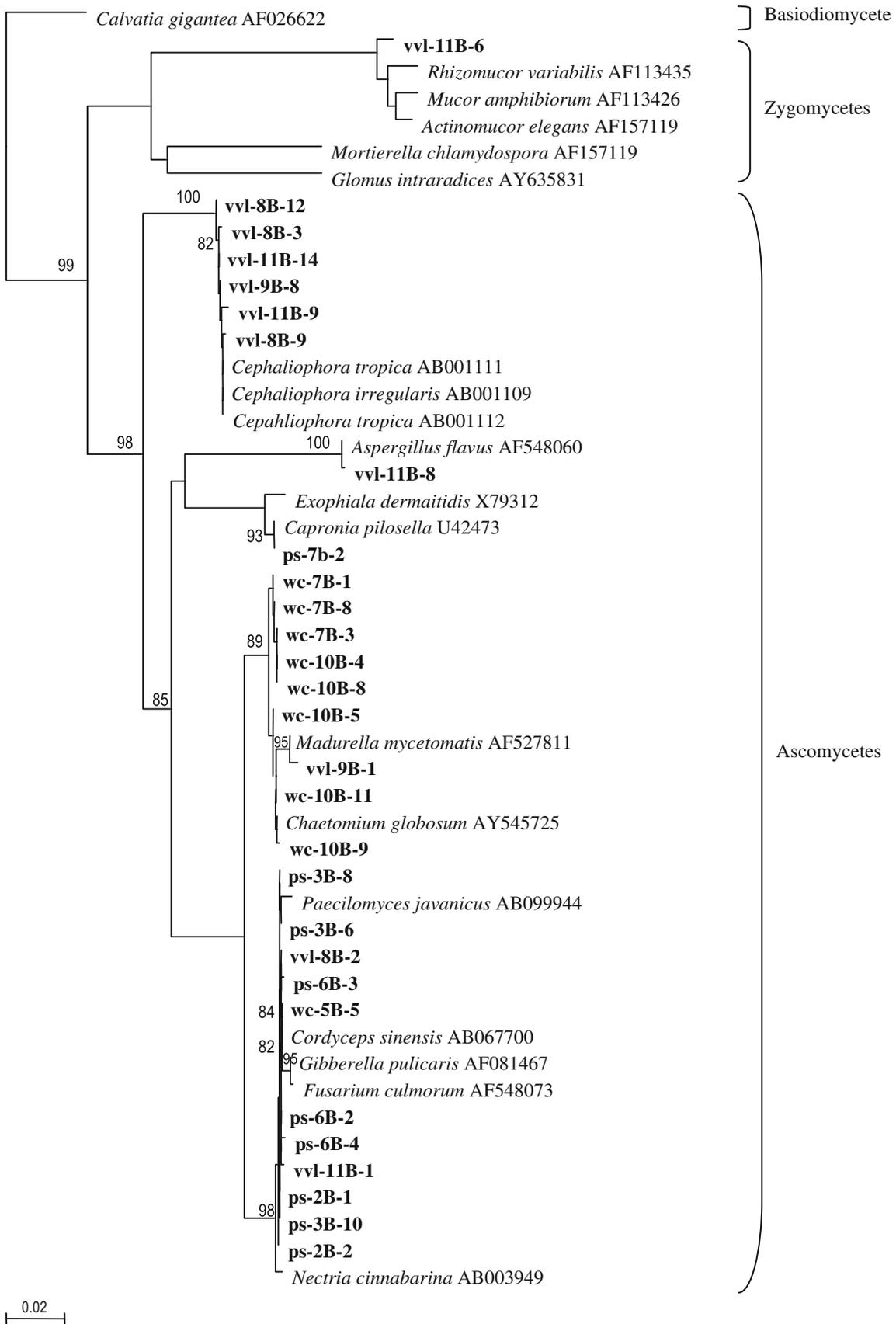
seeds showed a large proportion (99%) exhibiting varying extents of decay, presumed to be mediated by microbial activity, as uninoculated controls did not exhibit obvious signs of deterioration.

Discussion

Under conditions where seeds provided the majority of carbon nutrients, a predominance of fungal 18S rRNA gene sequences related to the *Ascomycota* were found in association with seeds, and further, certain fungal species were found more frequently in association with respect to seed type regardless of their soil origin. The ascomycetes include many saprobic species and known parasites of plant, animals, and other microbes (Thorn 1997). Zygomycetes are also known to be abundant in most soils with

many saprobic species able to grow rapidly in cultivation medium, often overwhelming the growth of other fungi present. Only one zygomycete-related sequence was obtained in this study, closely matching *Actinomucor elegans*, classified as a mucoraceous fungus found in soil and other natural habitats (Davel et al. 2001).

While care was taken in preparation of well-mixed microbial inocula, non-homogenous mycelial or spore dispersion may have resulted in unevenness of species distribution, thus affecting the outcome of potential encounters between seed and fungi. Nonetheless, the predominance of similar and specific ascomycete sequences found recurring on seeds with respect to three of the weed species in this study suggests the possibility of preferential associations that could occur depending upon the weed species and microbiota present. Visual examination of seeds indicated relatively dense microbial assemblages, suggest-



◀ **Fig. 2** Neighbor-joining tree showing the phylogenetic relationship of cloned 18 S rRNA gene sequences from seeds of velvetleaf (*vl*), Pennsylvania smartweed (*ps*), and woolly cupgrass (*wc*) to reference fungal taxa. Sequences used in the alignment ranged in sizes from 542 to 725 bp. The *scale bar* represents the number of base substitutions per site. Bootstrap values are shown for branch nodes that had >70% support in a bootstrap analysis of 1,000 replicates. The outgroup is the basidiomycete, *Calvatia gigantea*

ing further that microbial growth occurred at the expense of nutrients provided by the seeds themselves.

The fungal species found associated with woolly cupgrass seeds were dominated by close relatives of *Chaetomium globosum*, a common genus of soil saprophytes that are known to produce enzymes involved in plant residue degradation (Arora et al. 1991). *Cordyceps sinensis*, which was dominant in the clones recovered from Pennsylvania smartweed seeds, were also represented in a single clone each from woolly cupgrass and velvetleaf. Interestingly, species of *Cordyceps* are reported to be entomoparasites, and *C. sinensis* is known to produce bioactive substances and is used in herbal medicine to promote the immune system (Koh et al. 2003; Sasaki et al. 2004). While the ecological function of these fungi in relationship to weed seeds may only be speculated on at present, one hypothesis may be that fungi play specific roles related to preservation of seed hosts and thus contribute to seed bank longevity. Indeed, seeds of woolly cupgrass and Pennsylvania smartweed were previously found to have low susceptibility to microbial decay, in contrast to seeds of velvetleaf, which were highly susceptible to decay (Chee-Sanford et al. 2006). Fungi found associated with *Setaria* (foxtail) species were also found in previous studies to inhibit seed germination and possessed seed deterioration activity (Pitty et al. 1987).

A higher diversity of cloned fungal sequences was found in association with seeds of velvetleaf in this study. In a previous cultivation-based study conducted with velvetleaf seeds collected from the field, several species of fungi belonging to the *Ascomycota*, including *Penicillium* spp., *Fusarium* spp., *Trichoderma viridae*, *Verticillium* spp., *Gliocladium roseum*, and *Aspergillus* spp. were recovered, none having any apparent effect on seed viability (Kremer et al. 1984). The previous finding of majority *Ascomycota* associations with velvetleaf seed is consistent with the molecular-based results reported in this paper, with the predominant species found most closely related to *Cephalophora tropica*, a species common to a variety of soil (Al-Musallam 1990; Tanabe et al. 1999). The finding of multiple species of fungi on velvetleaf seed along with empirical observations of extensive seed deterioration suggest these fungal species may be associated with seed decay activities. Their role, or those of other fungi or bacteria, in initiating seed decay processes is yet unknown.

While a previous study showed a strong correlation between weed seed decay and soil microbial community composition (Davis et al. 2006), little is yet known about key specific populations that may be involved in seed deterioration processes and the environmental factors that drive that outcome.

Seeds represent a significant nutritional resource for soil microbial populations, and for this reason, microbial-mediated seed decay activities can occur. Velvetleaf seed coats reportedly produce a leachate containing aqueous-soluble phenolic compounds that exhibited phytopathic, anti-fungal, and anti-bacterial activities (Kremer 1986; Paszkowski and Kremer 1988). The higher mortality of velvetleaf seeds consistently seen in numerous instances in our laboratory studies suggest a high potential of natural microbial communities to overcome natural seed defenses for nutritional benefits to the microbes, given the opportunity for interaction to occur. While seeds may exert antagonistic effects on some members of the microbial community, certain weed species, such as Pennsylvania smartweed and woolly cupgrass, may host relationships that are nutritionally beneficial to microbes without mortal expense to the seed.

Results with seeds of giant ragweed, in contrast, did not yield extensive seed decay in comparison to what was observed in a previous study under field conditions (Davis 2007). Giant ragweed seeds, which include an outer protective woody involucre, may be structurally resistant to decay initiated by microbes, and access to seed nutrients might rely on the presence of more specialized members of the microbial community. Fungal 18S rRNA gene sequences were not recovered from two seeds showing visible colonization (Fig. 1d, h). One explanation may be that the primers used for amplification of fungal 18S rRNA genes did not detect species that were present or that fungi did not comprise a population density that was detectable using our chosen methods.

Whether the associations found in this study form naturally in soil is currently unknown. Conditions of spatial heterogeneity, patchiness in population distributions, and community composition among many other lesser-defined biotic and abiotic factors may all contribute to the interactions that may ultimately form between fungi and seeds. Shifts in fungal communities can occur seasonally (Daniell et al. 2001), and microbial diversity or populations tend to be higher in arable fields compared to natural or undisturbed soil (Buckley and Schmidt 2001; Daniell et al. 2001; Kirk et al. 2004). Plant community diversity also differs in similar fashion, which is also likely to be reflected in seed bank demography. In our study, we observed similar fungal species recurring with the set of agronomic soils (ASSET, ASLOC, and ASSWC) and the undisturbed, non-agronomic soil (USSEU). Further, there is diversity even

within these recurring species as indicated by higher resolution using ARDRA profiles. While many of the potential fungal interactions with seeds in soil may be primarily saprophytic in nature, the hypothesis that seeds can contribute nutritional benefits to soil-borne fungi with or without detriment to the seed itself can be further tested in the context of soil environments.

The potential biases typically associated with the use of molecular- and PCR-based techniques, such as sample size, and inefficiencies in cell lysis and DNA recovery are acknowledged in our study and may indeed have contributed to a seemingly low clone retrieval in our study. Recovery of DNA from single seeds, despite our best efforts, yielded low amounts from all seeds, and only 40–45% of the total number of seeds yielded amplified 18S rDNA. The use of the primer set EF4/EF3 was selected for its use in targeting a broad range of known fungi (Smit et al. 1999; Anderson et al. 2003; Anderson and Cairney 2004; Hunt et al. 2004), but its suitability for use in complete characterization of all the microbial species in seed associations is not yet known. While the use of these primers had conflicting reports of bias toward basidiomycetes and zygomycetes (Anderson et al. 2003; Smit et al. 1999), our use of this primer set did not reflect this nor did we obtain non-fungal sequences. Despite the possibility that the method might lack full coverage of the fungal assemblages, our results clearly indicated that the predominant cloned sequences recovered were *Ascomycota* and that similar sequences recurred within a seed type regardless of the source of microbiota. The higher resolution afforded by the use of ARDRA analysis allowed further details of the diversity among strains of highly related species and the directed relationship they may have with certain weed species.

Plants that are classified as weeds generally exhibit traits such as rapid growth, strong competitiveness, high fecundity under a wide range of environmental conditions, and produce long-lived seeds with regulated and discontinuous germination patterns (Booth et al. 2003). Weeds are often described as invasive plants and present as unwanted pests in both natural and managed landscapes. Besides the intrinsic attributes of plants that ascribe their invasive traits, there is currently little mechanistic information available on the potential importance of microbial associations in contributing to a plant's "weedy" character. This study characterized several species of the *Ascomycota* that formed associations with seeds of three common weed species and suggests that certain associations with a host seed may more likely occur even against a background of high complexity in population and environmental conditions usually present in soil. It provides the initial basis for further examining the relationships between soil fungi and seeds and examines a new aspect of weed seed bank

ecology. Within the context of weedy plants, these studies may further our understanding of seed bank dynamics and plant demography.

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